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16. ABSTRACT

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A CHROMATOGRAPHIC PERSPECTIVE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The coupling of high-performance liquid chromatography with mass spectrometry (HPLC–MS) is today a rapidly advancing field. In this paper, the chromatographic aspects of this coupling are reviewed. It is shown that the quantitative evaluation of resulting chromatograms, based on centralized second moment calculations of the bands rather than visual comparison with UV chromatograms, is required. The criteria for minimum influence on column performance by the interface and mass spectrometer are presented. Based on these principles, recent developments in HPLC column design are examined for their impact on HPLC–MS. Included in these developments are high-speed separations, as a consequence of small particle diameter (3 μm) columns and narrow tube diameter (micropacked) columns. In the latter case, the use of 200 μm fused-silica capillary columns with small particle diameter supports appears particularly promising. Finally, an assessment of the utility of post-column chemistries in HPLC–MS is given. The combination of these chromatographic developments with those of MS makes the future of HPLC–MS appear to be quite bright.

INTRODUCTION

The importance of coupling high-performance liquid chromatography (HPLC) and mass spectrometry (MS) is well-recognized today. The combination of the two techniques provides a powerful separation and qualitative/quantitative determination procedure[1,2]. Several years ago, a hotly-debated issue was —and to some extent, remains today— the question of on-line *versus* off-line coupling. Considering the relative ease of sample collection from an HPLC and the inherent difficulties of marrying two techniques that operate at pressures which differ by several orders of magnitude, proponents of the off-line mode have had strong arguments in their favor. In fact, the majority of users of HPLC–MS today operate in the off-line mode. However, it is also fair to say that most workers would prefer the on-line approach if the latter were well-developed.

Compelling arguments in favor of on-line HPLC–MS, relative to off-line coupling, include the following:

(i) convenience, especially when analyzing multicomponent mixtures;

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(ii) superiority in terms of speed of analysis;

(iii) reduced possibility for sample losses, particularly for trace level components;

(iv) ease of deconvolution of partially resolved chromatographic peaks because of the selectivity of the MS detector;

(v) reliable evaluation of chromatographic peak purity over the whole band, provided the sampling rate of the MS detector is compatible with the speed of elution of the chromatographic peak; and

(vi) accurate quantitation using isotopically labeled internal standards.

For these and other reasons, on-line HPLC–MS is clearly a desirable goal, which is approaching a realization as the interface designs for this combination are rapidly improving. Moreover, the fields of HPLC and MS are both undergoing dynamic growth. Developments in both techniques should ultimately lead to significantly more powerful HPLC–MS systems, which are able to attack problems that heretofore have defied solution.

Any discussions of HPLC–MS invariably requires a comparison with gas chromatography (GC)–MS. It is thus necessary to consider the relative merits of each method and the potential advantages offered by HPLC–MS. GC–MS is now an established analytical technique characterized by high sensitivity and resolving power. Trace analysis problems are now routinely solved by GC–MS using fused-silica capillary columns. On the other hand, the advantages of HPLC–MS arise from the inherent power of the liquid chromatographic column to elute substances which are not subject to molecular weight or volatility restrictions, and which do not require chemical derivatization for elution. As a consequence, the demands for preliminary sample cleanup and preparation for HPLC analysis are minimized. The wide variety of mobile phases and chemical equilibria which can be employed in HPLC also contrast dramatically with the limited selectivity manipulation available in GC.

On-line HPLC–MS involves the hybrid operation of two highly incompatible disciplines, much like the marriage of two different cultures (*e.g.*, C.P. Snow). In this marriage, it has been the tendency of the chromatographer to view the mass spectrometer as a sophisticated chromatographic detector while the mass spectroscopist has treated the high-performance liquid chromatograph as a sample delivery (inlet) system. Unfortunately, to a large extent, that situation still exists today in HPLC–MS and, when people approach the combination from a single perspective, they are likely to reduce the effectiveness of the system. Instead, it is our belief that a true hybrid system requires a total system approach, including sample clean-up considerations. With a view toward the integrated approach, appropriate compromises can be developed that utilize each component to its greatest extent.

In this paper, we will consider HPLC–MS as an integrated system from the chromatographer's point of view. General features related to the chromatographic performance of the HPLC–MS interface will be described, and recent trends in HPLC which, in our opinion, will impact strongly on HPLC–MS will be discussed. This paper in one sense updates a previous manuscript on this topic from the last Montreux meeting[3].

From the point of view of the chromatographer, the following factors are important for effective HPLC–MS operation: (a) freedom to select mobile phases and additives, (b) gradient elution, (c) no loss in separation from interface/detec-

tor/electronics, (d) quantitation (*e.g.*, linear dynamic range), (e) detection limits, (f) high-molecular-weight detection capabilities, (g) assessment of peak purity, (h) cost and operational simplicity.

Decoupling of mobile phase selection from the MS would allow the use of the full power of HPLC in terms of mobile phase additives[4]. In our view, gradient elution is essential for successful HPLC–MS operation, since the method is becoming increasingly important with the growing interest in the biological field, where mixtures covering a broad range of polarities are often found. Retention of chromatographic fidelity is also essential in HPLC–MS, *i.e.*, it is important to achieve the combination with minimal extracolumn variance. Today, the performance of an HPLC column is excellent, and it seems quite unsatisfactory to throw away part (or all) of this achievement from poor interface design.

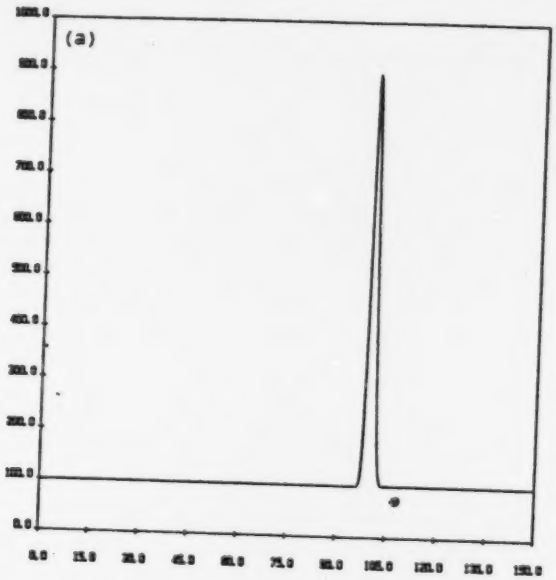
It is self-evident that if the HPLC–MS combination is going to become a routine analytical tool, the ability to carry out reproducible quantitative analysis with detection limits in the nanogram and picogram range will be essential. Furthermore, the ability to assess peak purity is one of the most powerful features of HPLC–MS as far as the chromatographer is concerned. What to the chromatographer may often appear as a pure chromatographic peak, may well contain several components; the selectivity of the MS detector provides a good opportunity for the deconvolution of unresolved doublets or multiplets. Moreover, for trace analysis, a wide linear dynamic range is essential. Finally, with regard to the detection of high molecular weight substances, it is interesting to point out the convergence of the goals of HPLC and MS. The separation of biopolymers is a field of increasing interest in HPLC[5]. Similarly, MS is now taking a direction towards the development of techniques and systems for the acquisition of spectra of compounds of high molecular weight, now approaching the 10,000 dalton range[6]. This common direction of HPLC and MS makes for a rather exciting prospect for the future development of HPLC–MS.

GENERAL CONSIDERATIONS

Evaluation of chromatographic performance of an HPLC–MS interface

An important consideration in an HPLC–MS interface is the maintenance of high chromatographic performance. Great advances have been made over the past decade in HPLC column design, and these advances are still continuing. Ever greater demands are being placed on the HPLC–MS interface (and the mass spectrometer). The characterization of the HPLC–MS interface will therefore become an ever more important consideration. In this section, we examine methods for characterization of the interface's influence on HPLC column performance.

When assessing the chromatographic performance of an HPLC–MS system, a common practice has been to compare visually the profiles of HPLC–MS and HPLC/UV chromatograms using identical columns and test mixtures. The fallacy associated with this approach is illustrated by inspection of the simulated chromatographic profiles of a pure peak, shown in Fig. 1. In Fig. 1A a chromatogram of $N = 10{,}000$ theoretical plates and with a perfectly symmetrical profile, *i.e.*, a gaussian peak, is shown. This example obviously corresponds to an ideal chromatogram. The second peak in Fig. 1B has a small exponential tail added to the gaussian of Fig. 1A, to simulate extracolumn effects. The peak has an asymmetry factor (*As*) of 20%. (*As*



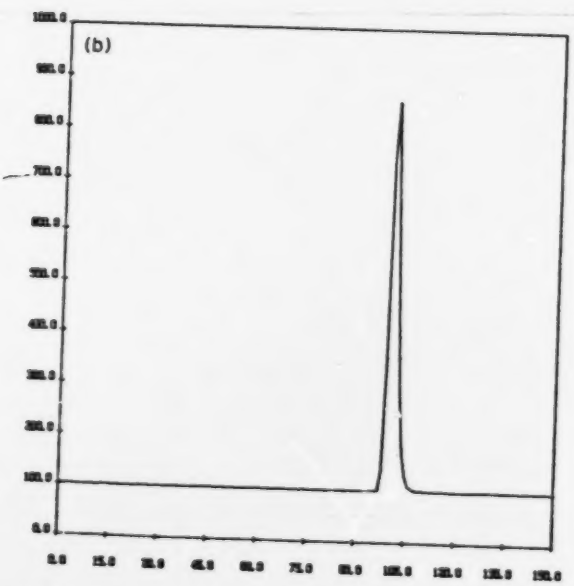


Fig. 1.

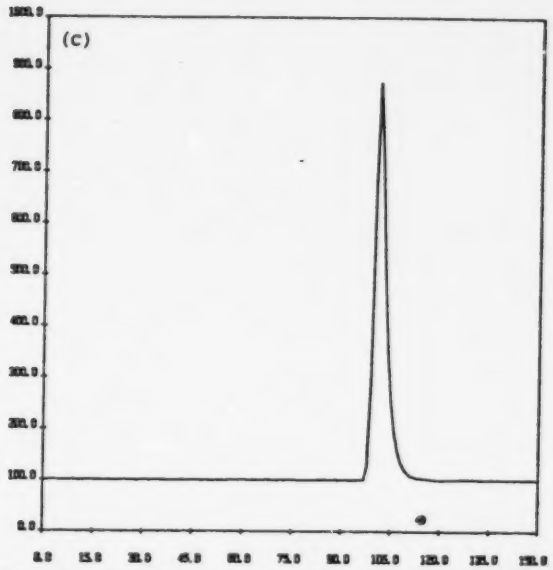


Fig. 1. Simulated chromatographic profiles to illustrate the influence of peak asymmetry on performance. (a) Symmetrical peak of N = 10,000 plates; (b) peak with asymmetry of 1.20, N = 5000 plates; (c) peak with asymmetry of 2.00, N = 2000 plates. For (b) and (c), an exponential tail is added to the peak in (a).

is defined as the ratio of widths on the forward and back sides of a perpendicular drawn from the peak maximum at 10% of the peak height, see Fig. 2.) Two points are noteworthy in Fig. 1B: (1) unless a worker has a highly trained eye, the two profiles of Fig. 1A and B appear very similar; (2) as indicated in Fig. 1B, a drop of 50% in plates is found, relative to Fig. 1A, and from $R_s \alpha N^{1/2}$ (R_s = resolution, N = theoretical plates), it can be calculated that a 30% lower resolution is obtained.

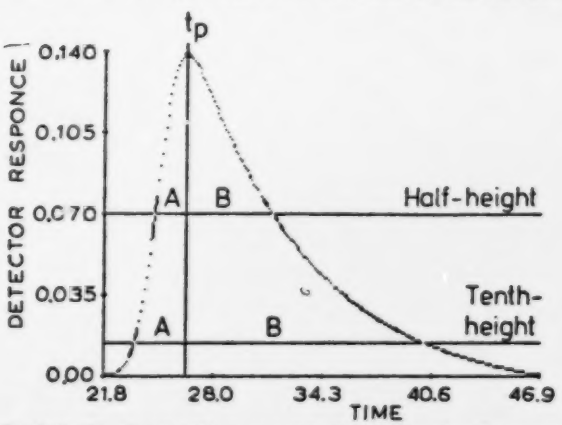


Fig. 2. Profile of peak with exponential tail indicating the points used to calculate the parameters in eqns. 5 and 6. Reproduced with permission from ref. 9.

The third profile (Fig. 1C) represents a peak in which the exponential tail yields an asymmetry of 2.0. The difference in profile shape between the first and third peaks is certainly discernible, although it might be tempting to suggest that, from a visual point of view, they are not really much different. Yet, as indicated on the figure, a five-fold loss in the number of theoretical plates is found, corresponding to a 55% decrease in resolution. From these examples, it can be concluded that mere visual observations are not valid for evaluating the chromatographic performance of an HPLC-MS system.

A more quantitative measure of chromatographic performance for a given HPLC-MS interface is clearly necessary. Frequently, workers characterize chromatographic profiles assuming symmetrical, gaussian peak shape. Unfortunately, in real life, chromatographic peaks are often not symmetrical, and the assumption of gaussian distribution can lead to significant errors in the estimation of peak widths. As is well known, the measurement of peak characteristics should be made using statistical moments (eqns. 1–3), where the second centralized moment corresponds to the correct variance of the peak. In these equations, the integrals have been replaced by summations, as this is the calculation method employed with computer derivation of the moments of a band.

$$M_0 = \text{area} = \Sigma h \tag{1}$$

$$M_1 = t_R = \frac{\Sigma ht - \Sigma h\bar{t}}{\Sigma h} \tag{2}$$

$$M_2 = \text{variance} = \frac{\Sigma ht^2}{\Sigma h} - t_R^2 \tag{3}$$

where h = peak height at time t; t = time; $\bar{t}$ = average time; t_R = retention time.

The importance of using moment calculations is further illustrated in the calculation of theoretical plates[7]. For an asymmetry factor of 1.3, assumption of a gaussian distribution introduces an error of +22%. This error becomes as high as +78% at an asymmetry factor of 2.1. Note that when gaussian shapes are assumed, the column appears to be significantly more efficient than it actually is.

Correspondingly, losses in resolution over that predicted also occur, as discussed in the examples of Fig. 1. The significance of this loss in resolution depends, of course, on the overall resolution. For a resolution greater than 1.5, *As* values of 1.5 or less may be acceptable (although not desirable), since separation of two bands will still be possible. If, on the other hand, the resolution of peaks is near 1.0 (closely spaced peaks), an *As* of 1.2 will affect the separation of the two bands. Since we are often interested in fast analysis, in which little time between the two components is required, consideration of peak asymmetry, even for relatively symmetrical peaks is thus important. In any event, peak moments must be used to characterize bands when asymmetry is evident.

Calculation of statistical moments from eqns. 1–3 requires a careful definition of the baseline, which is often difficult to achieve, particularly for peaks at low signal-to-noise levels. Errors in precision and accuracy will result when the baseline is not well-defined. In addition, a microcomputer evaluation of the chromatographic bands is required.

Two recent articles have proposed an approach for moment calculation to overcome in part these considerations. In the original work, Foley and Dorsey[8], using simulated chromatograms of peaks with exponential tails (the predominant case in chromatography), developed a series of empirical equations with the parameters indicated in Fig. 2 (*As* at 10% height). More recently, Anderson and Walters[9] modified this approach, where, instead of using the *As* at 10% peak height, they selected the *As* at 50% of the peak height. The latter method was shown to be effective up to *As* values as high as 5.0, as opposed to a maximum value of 2.76 of the earlier work[8]. The empirical equations for the calculation of the first and second moments based on the method of Anderson and Walters are given in eqns. 4 and 5.

$$M_1 = t_p + W_{0.5}\,[0.925 - 2.17 \exp(-0.848\, A_{0.5})] \tag{4}$$

$$M_2 = W_{0.5}^2/[1.06 + 54.0 \exp(-2.49\, A_{0.5})] \tag{5}$$

Experimentally determined values have been shown to be of high precision and accuracy. Of course, the chromatographic peak must be characterized as a gaussian with an added exponential tail. Nevertheless, the method can be used in a rather straightforward manner to calculate the moments and various features of a chromatographic peak, and for the evaluation of the true performance of the HPLC-MS interface.

Extra-column contributions to peak broadening

Once the proper characterization of the bands has been made, the next question is the influence of the interface on chromatographic performance. This evaluation involves a comparison of the column variance, σ_c^2, to the extracolumn variances, σ_{ec}^2. As has been often noted[10–12], the measured variance σ_T^2 on a recorder or data system is actually the summation of individual variance contributions.

$$\sigma_T^2 = \sigma_c^2 + \sum_i \sigma_{ec,i}^2 \tag{6}$$

The extra column variances can arise from various sources, *e.g.*, injector, capillary connections, etc. For purposes of this discussion, we will assume that these contributions are negligible compared to the interface contributions.

We can arbitrarily define acceptable extracolumn variance as 10% that of the column variance. Thus,

$$\sigma_{ec}^2 \leqslant 0.1\sigma_c^2 \tag{7}$$

or

$$\sigma_{ec} \leqslant 0.3\,\sigma_c \tag{8}$$

Above this level of contribution, unsatisfactory degrading of separation may occur. This can easily be seen in the following. The resolution between two components of equal peak width can be expressed as

$$R = \left(\frac{t_R}{\sigma_T}\right)^2 = \frac{t_R^2}{\sigma_c^2 + \sigma_{ec}^2} = \frac{N_c^{1/2}}{1 + \frac{\sigma_{ec}^2}{\sigma_c^2}} \tag{9}$$

With a σ_{ec}^2 contribution of 10% to σ_c^2, eqn. 9 reveals that a 10% loss in resolution will occur. As already noted, the actual impact of this effect will depend on the original R_s value.

Let us next turn to an examination of peak width. The peak width or variance of a chromatographic band is a function of the units employed. The length based variance (*e.g.*, in units of mm, μm), measures the width of the band in the mobile phase in terms of axial distance in the column. It is related to the height equivalent to a theoretical plate H as follows:

$$\sigma_L^2 = HL \tag{10}$$

Thus, the longer the column, the wider the band width. It is, however, important to recognize that σ_L^2 is relatively independent of k', the solute capacity factor. In other words, all peaks elute with roughly equal peak width, in terms of σ_L^2. Yet, when we monitor chromatograms with a recorder, we observe that in isocratic or isothermal operation the bands become broader with increasing k. This behavior is related to the fact that the recorder or data system, with a constant chart speed, is a time-based measurement.

The fundamental relationships associated with the time variance (peak width in time units) are summarized in eqns. 11 and 12.

$$\sigma_t^2 = \frac{\sigma_L^2}{(Rv)^2} \tag{11}$$

$$\sigma_t^2 = \frac{\sigma_L^2 (1 + k')^2}{v^2} \tag{12}$$

where Rv = band velocity, R = fraction in mobile phase = $(1 + k')^{-1}$. As can be seen in eqn. 11, σ_t^2 is equal to the length-based variance divided by the square of the band velocity. Since the band velocity is dependent on retention, $(1 + k')^{-1}$, it is apparent that σ_t increases with k'. In addition, σ_t becomes smaller with increased efficiency (large N), low k' or high mobile phase velocity, v.

The extra column variance in time-based units is a consequence of the response time τ_m of the detector (interface, mass spectrometer and electronics/data system). From eqn. 8, we can define an acceptable response time as follows:

$$(\tau)_m \approx \frac{0.3 t_R}{\sqrt{N}} \approx 0.3\sigma_t \tag{13}$$

$$\tau_m \approx \frac{0.3\sigma_L (1 + k')}{v} \tag{14}$$

Eqns. 13 and 14 will be used in a later section to estimate the response time of the detector necessary with state-of-the-art columns.

The third variance to consider is the volume variance, which can be written as

$$\sigma_v^2 = \sigma_t^2 F^2 \tag{15}$$

or

$$\sigma_v^2 = \sigma_L^2 (1 + k')^2 (\varepsilon_T \pi r^2)^2 \tag{16}$$

where F = mobile phase flow-rate, ε_T = total column porosity and r = tube radius. Note that the volume variance is related to the fourth power of the tube radius, see eqn. 16. It is also useful to recognize that the peak volume can be approximated as 4 σ_v, for bands approximating a gaussian shape. As we will discuss later, miniaturization of column dimensions places great demands on minimization of extracolumn volume variance.

Principles of detection

Chromatography is fundamentally based on selective dilution of the solute species by the mobile phase, the later eluting species being more dilute than the earlier ones. If we assume a gaussian distribution, the equation for concentration at the peak maximum upon elution is related to the mass of sample injected, m, the efficiency of the column, N, and the retention volume, V_R, by

$$C^{max} = \frac{mN^{1/2}}{(2\pi)^{1/2} V_R}. \tag{17}$$

We can immediately see that the concentration at peak maximum varies linearly with the mass of material. In addition, the more efficient the column, the greater the signal, whereas the larger the retention volume, the smaller the signal. This latter point means that, for a given number of theoretical plates and quantity of sample injected, C^{max} will be higher for species with smaller k' values.

In chromatography, we can categorize detectors generally in terms of their response characteristics, as either being concentration sensitive or mass flow sensitive. In HPLC today most detectors, *e.g.*, UV, fluorescence, are based on concentration. However, the mass spectrometer is strictly a mass flow sensitive device. It should be noted that none of the current modes of HPLC–MS coupling alter this aspect of the mass spectrometer. In a mass-flow sensitive detector, the signal can be expressed as

$$\frac{dm}{dt} = C^{max} \cdot F \cdot S \tag{18}$$

where S = splitting ratio. Since the flux of material reaching the detector per unit time is proportional to the flow-rate, higher flow-rates yield higher signals. Of course, the maximum flow-rate will depend on the interface design and mass spectrometer specifications. Note also that when the flow-rate is constant, the mass-flow sensitive detector is directly proportional to concentration.

RECENT DEVELOPMENTS IN HPLC

Having considered some basic principles in the coupling of HPLC with MS,

we will now turn to a survey of recent advances in HPLC which will undoubtedly enhance the power of HPLC–MS. These advances include, first, packings with particle diameters less than 5 μm for high-speed separation and secondly, narrow bore columns, especially 200–300 μm fused-silica capillaries, for high-performance operation with low mobile phase volumetric flow-rate. We will then turn to some aspects of detection limits, both in general and in terms of current column development. Finally, we will consider the potential of post-column chemistries interposed between the column and interface to increase the analytical capabilities of HPLC–MS.

Small particle diameter columns

In the early 1970's, column packings with 20 μm particles were the state of the art. Today, the standard is about 5 μm, and there are commercially available columns with 3 μm particles. In a recent article, Dewaele and Verzele[13] reported the successful preparation of 2 μm particle columns.

If a well-packed column can be maintained as the particle diameter d_p is decreased, then a common equation of reduced plate height, h, *vs.* reduced velocity, ν, will be obtained which is independent of d_p[14]. The generally accepted relationship can be written as

$$h = B/\nu + A\nu^{1/3} + C\nu \tag{19}$$

where A, B and C are constants, $h = H/d_p$ and $\nu = \upsilon d_p/D_m$, H = plate height, d_p = particle diameter, υ = mobile phase velocity and D_m = solute diffusion coefficient in the mobile phase. This equation yields a minimum h, ($h_{min} \approx 2$) and optimum reduced velocity ($\nu_{opt} \approx 5$), which will be common for all particle sizes, again if the columns are well-packed.

From the constant ν_{opt} and the definition of ν, it is clear that as the particle size of the packing is reduced, υ_{opt}, the optimum mobile phase velocity, must increase proportionately, in order to maintain the optimum reduced velocity constant. Secondly, as d_p is reduced, H_{min} will be lower, if h_{min} is constant. Hence, for a given plate count, N, a shorter column can be used ($H = L/N$) with a packing of lower d_p. The decrease in H_{min} and the increase in ν_{opt} lead to the conclusion that smaller d_p particles will yield faster analyses, other things being equal. This speed factor is further emphasized if velocities greater than optimum are used[15]. An example of rapid separation using a 3-μm particle column is illustrated in Fig. 3, which shows the resolution of 20 phenylthiohydantoin (PTH)-amino acids in 10 min[16].

Fast analyses are important when HPLC–MS is considered, since the more rapid the separation, the more efficient is the use of the mass spectrometer. Given the complexity and expense of the mass spectrometer and the potential for downtime, high-speed chromatography is therefore clearly advantageous. The question that needs to be asked, however, is, will we reach a point where LC is too fast for MS[17]? To answer this question, we need to understand the current demands placed on MS and the expected future demands. What, if any, constraints are being placed on present MS instrumentation?

In order to examine this, let us assume a column of 15 cm length packed with 5-μm particles, yielding N = 10,000 (typical for a commercial reversed-phase LC column). For a peak with a retention time, t_R = 5 min, we can calculate,

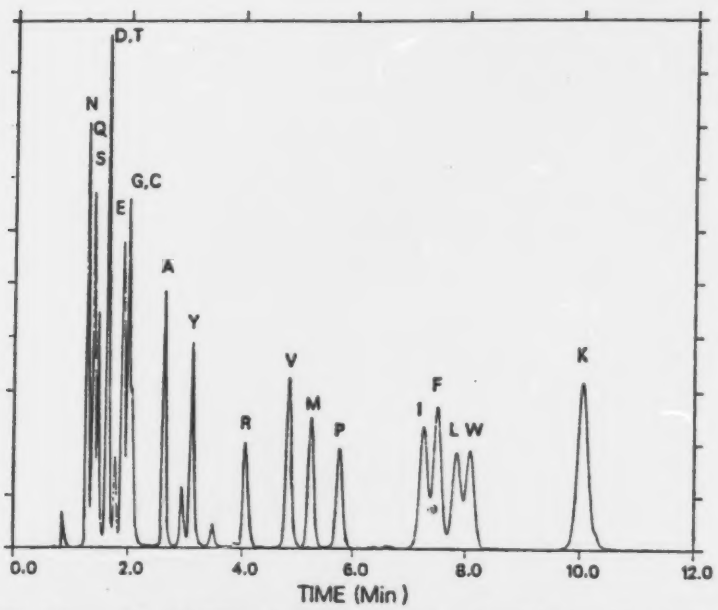


Fig. 3. Separation of 20 PTH-amino acids; 3 μm Zorbax phenethyl column (8 × 0.62 cm); flow-rate 2.0 ml/min; mobile phase: acetonitrile–12 m*M* phosphate buffer (pH 3.2) (36:64). Compounds: A = alanine; C = carboxymethylcysteine; D = aspartic acid; E = glutamic acid; F = phenylalanine; G = glycine; I = isoleucine; K = lysine; L = leucine; M = methionine; N = asparagine; P = proline; Q = glutamine; R = arginine; S = serine; T = threonine; (T) = dehydrothreonine; V = valine; W = tryptophan; Y = tyrosine. Reproduced with permission from ref. 16.

based on eqn. 13, an acceptable time constant $(\tau)_m \leqslant 0.9$ sec. On the other hand, if t_R is reduced to 30 sec, $(\tau)_m \leqslant 0.09$ sec. Thus, for "typical" high-performance columns, detector response times of 1 sec or less are required. For high-speed columns, response times less than 0.1 sec will be necessary.

Let us now consider the meaning of these required response times in terms of HPLC–MS practice. In order to assess peak purity, it is estimated that 4–5 points across a peak, corresponding to one point for every standard deviation, is necessary[15]. Based on eqn. 13, a σ_t value of 3 sec is required for minimum peak distortion, with a detector τ of 0.9 sec, and correspondingly 0.3 sec for a τ of 0.09 sec. Thus, for peak purity assessment with fast columns, a whole spectral scan may be required in as little as 0.3 sec or less. When the mass spectrometer is operated in the scanning mode, a rate of 0.3 sec/scan or higher may therefore be needed. Indeed, in the future, scan rates of less than 0.1 sec/scan may well be necessary. Even 0.3 sec/scan is a rather prohibitive demand, since the fastest scanning magnetic mass spectrometers barely approach rates of 1.0 sec/scan. Moreover, quadrupole mass spectrometers are not normally operated at rates much below 0.5 sec/scan. Despite the fact that electron multiplier detectors have sufficiently low time constants (for an input resistor $R_i = 10^8\ \Omega$ and a ground capacitance, $C = 10^{-12}$ F, $\tau = 10^8 \cdot 10^{-12} = 10^{-4}$ sec), loss of resolution and sensitivity inhibit such rapid spectral acquisition[18]. Turning next

to the issue of quantitation, approximately 10–20 points per chromatographic peak are required, in order to be assured of recording the peak maximum. Even though *a priori* this may seem even more of a problem than peak purity assessment, the severity of the problem is reduced by the fact that most quantitative analyses are conducted in the selective ion recording mode or by scanning a limited mass range. Nevertheless, it is fair to conclude that we are challenged today in MS to acquire data at a more rapid rate[17].

One answer to this challenge may be provided by Fourier transform mass spectrometry, FT-MS, where such rapid scanning is, indeed, possible[19]. The application of FT methods is now revolutionizing MS as it did for both IR and NMR. The primary advantage of FT methods for rapid data acquisition is the simultaneous detection of all masses in the spectrum. Indeed, FT-MS instruments are able to record the entire mass spectrum in the time required by conventional scanning instruments to record a single mass. Currently, commercial FT instruments can generate up to 200 low-resolution spectra per sec, 100 medium-resolution spectra per sec, 10 high-resolution spectra per sec and an ultra-high-resolution spectrum in less than a sec over a mass range of greater than 3000 daltons[20].

Unlike conventional scanning spectrometers, ion detection in FT-MS is not based on formation of ions in a "messy" ion source and the extraction of these ions into a separate mass analyzer. Instead, ions have traditionally been formed and analyzed in the same region. This approach has placed severe limitations on sample introduction techniques available to FT-MS. The desire to apply the high data acquisition rates, high resolution and high mass range capability to analytical separation techniques is, however, pushing the development of FT-MS in the area of sample introduction techniques. The recent appearance of a differentially pumped dual cell commercial FT-MS with separate regions for ion formation and detection has removed many of the sample introduction limitations of this technique. Commercial FT instruments are now available that allow routine medium to ultra-high resolution GC–MS studies, and research is in progress to couple HPLC to FT-MS[21].

Narrow-bore columns

The second trend in HPLC which is important for HPLC–MS is the development of narrow-bore high-efficiency columns.[22] In the early days of HPLC, 2 mm I.D. columns were typical; however, today, 4.6 mm I.D. analytical columns are generally used. Recently, microbore columns of 2 mm I.D. and 1 mm I.D. have become widely available, and most recently, micropacked columns using fused silica capillaries of 200 μm I.D. with particle diameters of 3–5 μm have also been successfully developed[23,24]. Research into the development of very narrow-bore (5–30 μm) capillary HPLC is also in progress[25]. However, many advances will be required before capillary HPLC is a practical tool, and we will therefore focus in this discussion on packed columns.

The implications of the trend to narrow-bore packed columns for HPLC–MS become immediately apparent when we consider the corresponding decrease in mobile phase flow-rate (relative to normal-bore columns), since, for the same velocity, the flow-rate is proportional to the square of the column diameter. Especially interesting is that the 200-μm columns operate at liquid flow-rates approaching 1 μl/min, corresponding to gas flows of *ca.* 1 ml/min. This gas flow-rate is equivalent to that

found in capillary GC–MS and, as a result, the fused-silica micropacked columns can be attached directly into the mass spectrometer ion source for electron impact or chemical ionization with the reagent gas of choice. The feasibility of this approach in HPLC–MS has already been demonstrated at Montreux[26]. Interestingly, the authors of this work also report the acquisition of spectra even with the filament off, indicating the possible occurrence of thermospray ionization phenomena.

Microbore and micropacked columns are obviously most compatible with HPLC–MS interfaces which function best at low flow-rates. Typical examples are the direct liquid introduction (DLI)[27], the moving belt interface[28,29] and the electrospray method[30]. However, these narrow-bore columns may prove to be useful even for the thermospray interface which appears to work best at flow-rates of approximately 1–2 ml/min. It was previously shown[31] that the effluent from a capillary column could be swept through a capillary orifice and into the mass spectrometer without loss in column performance by maintaining a constant flow of a sweep solvent. The advantages of this approach for thermospray HPLC–MS are described later in this paper.

It must be recognized that the use of the narrow-bore columns can create instrumentation demands related to the need for careful control of the low flow-rates and miniaturization of the injector and interface/detector volumes. Injection problems can be often overcome by injecting the sample in a larger volume of a weaker solvent than the mobile phase, thus achieving trace enrichment or sample focussing, similar to on-column injection in GC. Of course, this is also readily achieved in gradient elution[32]. A more serious concern, however, can be the difficulties associated with the miniaturization of the interface/detector. The low volumes of the eluted bands demand low detector volumes to compensate for band broadening arising from σ_v^2, the volume variance (see eqn. 15 or 16).

In this regard, it is interesting to calculate the volume-based variance of selected chromatographic peaks for different diameter columns (Table I). Note that all examples involve columns with the same size particles (5 μm). For the 4 mm I.D. columns, peak variances in excess of 100 μl^2 can be calculated, even with $k' = 0$. On the other hand, for a 200 μm I.D. column, even at $k' = 4$, the peak variance is only 0.12 μl^2. These numbers illustrate the potential constraints that are imposed in interface/detector design for HPLC–MS with 200-μm columns. Nevertheless, successful coupling is definitely attainable in HPLC–MS. We have already noted previous success with capillary column LC for $k' = 0$[31], and it is worth mentioning that at the

TABLE I

VOLUME-BASED VARIANCES CALCULATED FOR A VARIETY OF COLUMN CONDITIONS

$h = 2.5$, $d_p = 5$ μm, $\varepsilon_T = 0.70$. Note: For no extra column effect, $\sigma^2_{v,ec} \leq 0.1\sigma^2_{v,a}$ or $\sigma^2_{v,ec} \leq 0.3\sigma^2_{v,a}$.

k'	dc (mm)	L (cm)	σ_v^2 (μl^2)	σ_v (μl)
0	4	15	140	12
2	4	15	1260	36
0	0.4	100	0.1	0.30
0	0.2	100	0.005	0.07
4	0.2	100	0.12	0.35

Montreux meeting peak volumes of the order of 1 nl in directly coupled capillary HPLC MS were reported using a sweep liquid-interface design[33]. Clearly, the fact that the mass spectrometer is a mass flow sensitive detector, thus permitting sweep liquids without loss in signal, is advantageous for miniaturized columns.

On another point, plate counts for the micropacked (3–5 μm particles) columns can be of the order of several hundred thousand theoretical plates, or more. An example of high resolution is illustrated in Fig. 4, which shows the separation of a standard mixture of benzoylated steroids on a 1 m × 0.24 μm I.D.column. Efficiency in excess of 100,000 theoretical plates is observed[34]. Note, for example, the separation of closely related steroids such as 11-hydroxyandrosterone and 11-hydroxyetiocholanolone (peaks 1 and 2). Typical analysis times, however, can be of the order of several hours, as shown on the figure. Longer analysis times than in Fig. 4 can be observed when longer columns (with corresponding larger plate counts) are employed. There are certainly complex samples where columns in excess of a few hundred thousand plates are important; however, the number of such samples will be clearly limited.

The goal of achieving 500,000 to 1,000,000 theoretical plate columns is of current interest. However, for such ultra large plate count columns, not only is analysis time of concern, but also the reproducibility both in terms of run-to-run and column-to-column. A column with $N = 10^6$ can achieve baseline resolution for a pair of solutes with a relative retention (α) value of 1.0015. While at first glance this value looks spectacular, the demands in terms of mobile phase repeatability, column constancy from run to run, not to mention the packing of a new column with a new batch of silica, would appear significant. At the very least, columns with $N \gg 500{,}000$

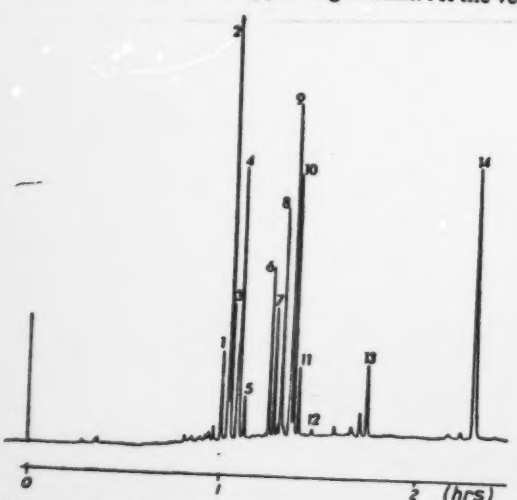


Fig. 4. Separation of 14 benzoylated steroid standards. Stepwise gradient conditions: acetonitrile–water (80:20) (0.5 min); acetonitrile–water (85:15) (14 min); acetonitrile–water (90:10) (18 min); 100% acetonitrile. Other experimental conditions are described in the text. Key: (1) 11-hydroxyandrosterone; (2) 11-hydroxyetiocholanolone; (3) allotetrahydrocortisol; (4) tetrahydrocortisol; (5) tetrahydrocortisone; (6) β-cortolone; (7) β-cortol; (8) α-cortolone; (9) α-cortol; (10) etiocholanolone; (11) androsterone; (12) dihydroepiandrosterone; (13) pregnanetriol; (14) androstandiol. Reproduced with permission from ref. 34.

require detailed examination of reproducibility. It seems that perhaps a more practical goal is ≈100,000–200,000 plates for high resolution. Such columns could be powerfully coupled to a mass spectrometer for analysis of highly complex samples.

Analyte detection and sensitivity

The recent improvements in HPLC-MS technology have also increased the expectation and demand for lower detection limits. For all of the common modes of HPLC-MS coupling (*e.g.*, thermospray, DLI, moving belt, electrospray) sensitivities in the picogram range have been reported in selected examples. The detector response to a given amount of analyte is reflected in the signal-to-noise ratio. In this section, we examine some of the parameters which influence this ratio from the HPLC end of the HPLC-MS coupling in conjunction with the new trends in HPLC. We will begin with the background noise.

If we disregard considerations such as sample delivery to the ion source, method of ionization, ion transmittance in the mass spectrometer and electrical noise, a major limiting factor to lower detection limits in HPLC-MS appears to be chemical noise. This source of noise tends to vary from one mode of HPLC-MS coupling to another. For example, in thermospray HPLC-MS the chemical noise occurs mostly in the form of solvent ion clusters which may extend into the 200 amu range[35], while in the moving belt interface, chemical noise is prevalent at m/z values less than 100, depending on the mobile phase[36]. Results from this laboratory with the moving belt interface illustrate the significance of chemical noise to detection limits. For polynuclear aromatics (PNAs) of MW ≈ 300 daltons, detection limits as low as 40 pg were achieved[37]. On the other hand, our experience with related molecules (*e.g.*, substituted phenols) with a molecular mass of 90–130 dalton was that, at best, 1 ng was the limit of detection[29].

An important consideration as we strive for lower detection limits is the recovery of material from the chromatographic column. This point is particularly relevant for the analysis of highly polar, nonderivatized species by HPLC-MS. A potential danger exists for the loss of polar substances, either by the packing material, frits, or column tubing. Recent work on iodinated peptides indicates that recovery can indeed be high, even at very low samples levels[38]. Nevertheless, such factors as the quality of bonded ligand coverage to a silica surface, the specific ligand and type of silica employed, and the role of metal on the silica surface[39] will become highly critical in ultratrace analysis. As detection limits improve, it will be necessary for the chromatographer (and mass spectroscopist) to pay greater attention to recovery from a column.

Another factor which also merits consideration, when we operate in ultratrace analysis, is the purity of the mobile phase components. The so-called "HPLC-grade" solvents used today are generally purified to remove UV active impurities. However, the mass spectrometer is a universal detector and, hence, chemical background noise from the solvents, particularly at trace levels, can limit detection. This means that new or extended approaches to solvent purification may be required in the future for ultratrace analysis by HPLC-MS. Under such circumstances, it is likely that the cost of these solvents will increase from their present levels. Thus, for low level detection, the narrow-bore columns, discussed earlier in this paper, because of the low flow-rate involved, could be advantageous.

Let us consider next the characteristics of the mass spectrometer as a detector, and the implication of the trend towards narrow-bore HPLC columns on the performance of the mass spectrometer. We have already noted that the mass spectrometer is a mass-flow sensitive device. It is clear from eqn. 18 that any splitting of the flow after the column to accomodate the liquid into the mass spectrometer, *e.g.*, for the DLI[27], can markedly decrease the signal from the detector. With narrow-bore columns, the complete flow can enter the mass spectrometer, which is advantageous. In our work with the moving belt interface[29], we also found the low flow-rates from a microbore column useful, especially our wide mobile phase composition gradients. As shown in Fig. 5, using a 1 mm I.D. column with nebulation for deposition of the effluent on the belt, a mixture of phenols from a coal gasifier condensate sample could be analyzed by electrom impact MS with a wide gradient range.

In the case of a concentration sensitive device, it is clear from eqn. 17 that for a given mass of injected material, the narrower the column tube diameter, the larger will be the value of C^{max} (as a consequence of a decrease in V_R). Thus, in cases where the amount of sample is limited, a clear potential exists using narrow-bore columns with concentration sensitive detectors. This concentration enhancement is not available for a mass-flow sensitive device, since C^{max} and F are inversely related to the tube diameter, see eqn. 18. However, it frequently occurs that, in the miniaturization of the detector for a concentration sensitive device, the response characteristics decrease (*e.g.*, a decrease in the path length of the detector cell for a UV detector), whereas no loss in response is obtained with a mass-flow sensitive device.

Let us next discuss the coupling of a narrow-bore column with MS using the thermospray approach. As we noted earlier, thermospray ionization has proved effective at flow-rates in the range 1–2 ml/min. In order to conduct thermospray HPLC–MS with narrow-bore columns, investigators have considered the use of a sweep liquid of ammonium acetate buffer into the thermospray device. While this approach may introduce a dilution factor which can reduce the signal C^{max}, the higher flow-rate compensates for that reduction and cancels the negative effect from F (see eqn. 18). This procedure is often followed when coupling capillary GC columns to the mass spectrometer, in which case a sweep gas is used. The approach is indeed interesting, because, as discussed at the workshop, it effectively decouples the high-performance liquid chromatograph from the mass spectrometer since the main solvent going into the mass spectrometer is the sweep liquid. As a consequence, any modifier of choice in the mobile phase, such as metals, buffers, or other additives, could be used. The detector could be optimized to the sweep solvent alone, an important feature in thermospray ionization where ion formation is strongly influenced by the solvent, temperature, etc.[40]. In addition, as we extend to lower detection limits, we would not have to worry about the purification of all solvents, but simply concentrate on the purity of the sweep liquid. The monetary savings could thus be a significant factor. In many respects, therefore, micropacked thermospray HPLC–MS with a sweep liquid offers some intriguing possibilities.

Post-column chemistry

Post-column chemistry in HPLC has been used with a variety of detectors, including fluorescence, UV, etc.[41]. In effect, this approach also provides a means of decoupling the chromatograph from the detector by permitting the analyst to carry out the HPLC separations with the intact analytes, while the post-column reaction

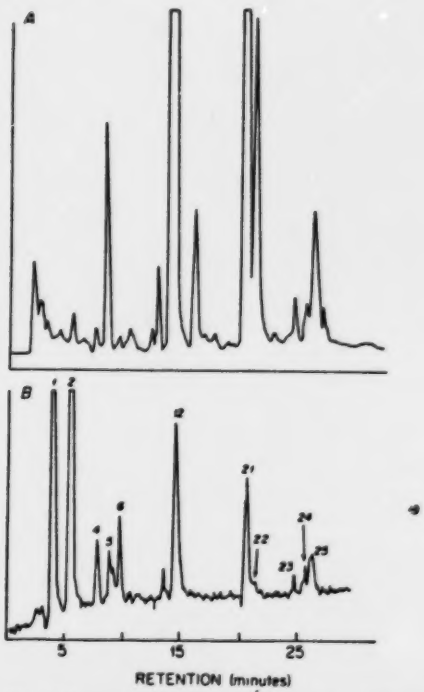


Fig. 5. Separation of a coal gasifier condensate sample using 35 cm × 1 mm, C_{18} microbore column. Solvent A (5% acetonitrile, 5% H_2O, 0.1% TFA); gradient 5–70%B in 40 min. Flow-rate 100 μl/min (a) HPLC-UV, 280 nm; (b) HPLC-MS, electron impact, moving belt interface. Reproduced with permission from ref. 29.

can be used to increase sensitivity and/or selectivity for specific analytes, compatible with the detector on hand. Note that in GC–MS extensive cleanup and precolumn derivatization may be required in order to elute substances through the column. In post-column chemistry-HPLC–MS, the advantage of limited sample clean-up of HPLC relative to GC is maintained.

In our laboratory, we have employed post-column chemistry during HPLC–MS in a variety of ways, including ion-pair extraction[36], Schiff base formation and extraction and alkylation[42]. This work was done with the moving belt interface, which appears readily adaptable to post-column HPLC–MS. Moreover, one of the advantages which can be realized with post-column extraction techniques in reversed-phase HPLC–MS is an improvement in detection limits because of the reduction of chemical noise.

In ion-pair HPLC–MS further contribution to chemical noise may arise from the spectral overlap of the counterion and the solute moieties. This can be minimized by judicious selection of the type of counterion, taking into consideration the structural features which govern the fragmentation of organic compounds in mass spectrometry[36]. This is illustrated in Fig. 6. Aromatic counterions, which would not generally be used in HPLC–UV, provide a suitable alternative for HPLC–MS.

Another interesting example of post-column chemistry in HPLC–MS is the use

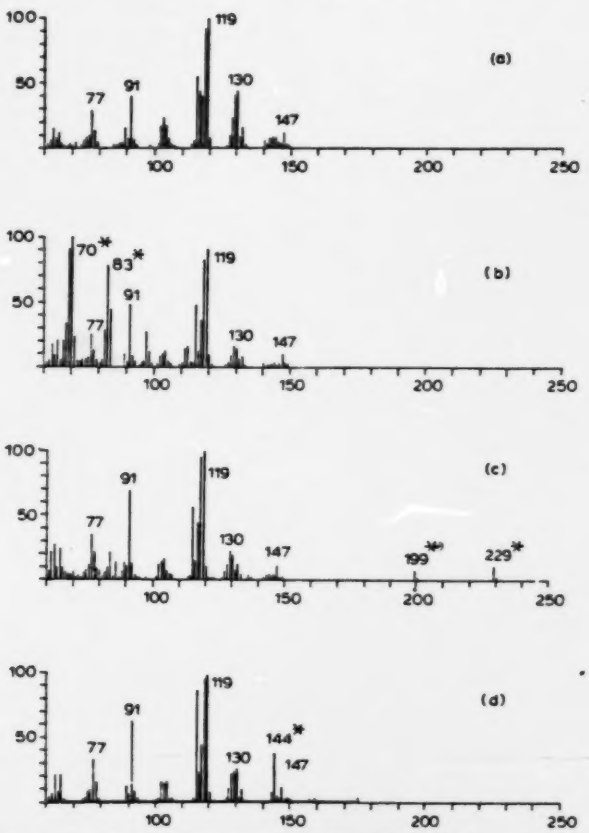


Fig. 6. Electron impact mass spectra of (70eV) (a) α-methylparnate; (b) α-methylparnate–C_{10} alkylsulfate ion pair; (c) α-methylparnate–picrate ion pair; (d) α-methylparnate–naphthalenesulfonate ion pair. Reproduced with permission from ref. 36.

of selective enzymatic cleavage of peptides with the thermospray interface[43]. This approach provides additional peptide fragments, which can be subjected to mass analysis in order to "piece" together the original peptide. This approach is simplified by the fact that thermospray mass spectra are dominated by molecular ion related adducts. Finally, a paper at the Montreux meeting reported on the interfacing of a micro LC (0.7–1.0 mm I.D. column) system with a DLI HPLC–MS unit via a post-column continuous liquid–liquid extractor. Aromatic hydrocarbons were analyzed by normal extraction, while acidic organic pesticides were extracted using ion pairing techniques[44].

The positive features of post-column chemistry notwithstanding, it is also reasonable to point out that the approach is not general. It is probably fair to note that post-column chemistry in HPLC–MS will not be turned to for all analyses. This is primarily because the approach introduces an additional step into the HPLC–MS analysis, albeit without significant extracolumn band broadening contributions. Whether a post-column (or precolumn) approach becomes adopted by a laboratory

will depend on the number of samples of a given type which must be analyzed, and how routinely the specific analysis is being conducted. Under routine conditions, post-column techniques can prove to be advantageous.

CONCLUSIONS

On-line HPLC–MS has now entered into its second decade. The progress which has been achieved, although slow, is nevertheless remarkable. Beginning with a discontinuous sampling system using an automated probe[45], the technique advanced to a truly on-line system, but one which utilized less than 1% of the HPLC effluent[46]. Virtually complete sample utilization was eventually realized with microbore columns using direct liquid introduction[27], and improved continuous transport (moving belt interface)[37] and, finally, with the thermospray ionization system which permits the introduction of HPLC effluent into the mass spectrometer at flow-rates as high as 2 ml/min[2]. Thus, despite earlier predictions of the impossibility of this coupling, it is fair to say that HPLC–MS has come of age. Nevertheless, much remains to be accomplished before it can be considered a routine analytical technique.

In trying to project the future growth of HPLC–MS it would be interesting to reflect back and compare it to the development of GC–MS. In the early days of GC–MS, packed columns operated at flow-rates of 20–30 ml/min and required a splitter or enrichment device. The development of capillary columns and fast scanning mass spectrometers effectively eliminated the need for these devices or, for that matter, of any interface for GC–MS. HPLC–MS has started essentially on the same basis. The trend, however, toward narrow-bore high-efficiency HPLC columns, coupled with improved vacuum designs, extended mass range and ionization methods in MS (*e.g.*, thermospray, fast atom bombardment, laser desorption, electrospray), and new techniques for mass analysis (*e.g.*, MS–MS, FT-MS) hold considerable promise for the eventual simplification of the technology of HPLC–MS coupling and its adoption as a routine analytical method. It is our hope that the considerations in this paper will be helpful towards the design of an optimized integrated system capable of maintaining maximum chromatographic performance.

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